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Synthesis, stereochemical assignment, and bioactivity of the *Penicillium* metabolites penicillenols B_1 and B_2

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ABSTRACT

The *Penicillium* metabolites penicillenols B_1 and B_2 were synthesised for the first time by elimination of a mesylated penicillenol A precursor as obtained from an L-threonine derived tetramic acid. The (*Z*,*S*)- and (*E*,*S*)-configured diastereomers were identical to the natural compounds as to NMR spectra and optical rotations. Both isomers showed antiproliferative effects against cancer and endothelial cell lines and penicillenol B_1 was also notably antibiotic against *Staphylococcus aureus*.

1. Introduction

The penicillenols **1** were isolated from *Penicillium* sp. GQ-7, an endophytic fungus associated with *Aegiceras corniculatum*.[1] They are *N*-methylated pyrrolidine-2,4-diones (tetramic acids)[2] bearing an α-methyl branched C_8-fatty acyl residue at C-3 (Fig. 1). The penicillenols A_1 **1a** and A_2 **1b** were synthesised by Yoda et al. via cyclisation of *N*,*O*-protected L-threonine and D-allothreonine, respectively, with Meldrum's acid and subsequent 3-acylation of the so-formed tetramic acids.[3] Our group reported the synthesis of penicillenol C_1 **1e** via Wittig cyclisation of a protected benzyl L-*N*-methylthreoninate with the phosphorus ylide Ph_3PCCO **2**.[4] In either case the comparison of the NMR and optical rotation data of the synthetic products allowed the configurational assignment of the natural products. In contrast, the penicillenols B_1 **1c** and B_2 **1d**, which are formal dehydration products of penicillenol A have neither been synthesised nor had their configuration at C-9 been assigned.

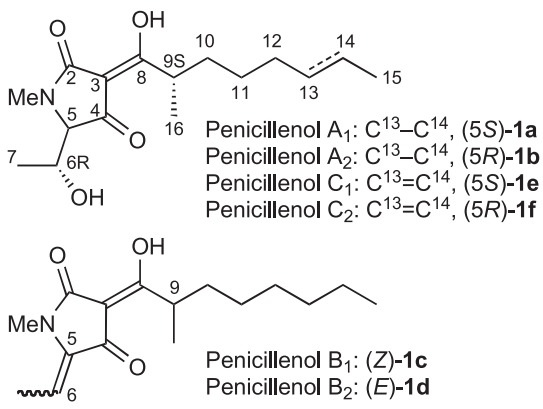

Fig. 1. Structures of penicillenols **1a**–**f**.

Herein we report the first synthesis of **1c** and **1d** employing an alternative third route to the threonine derived tetramic acid core, and the assignment of the configuration of C-9 of the natural penicillenols B_1 and B_2. Since they had shown antiproliferative activity against HL-60 leukaemia with single-digit micromolar IC_{50} values we also assessed their efficacy against further cancer and endothelial cell lines. As a first test for antibiotic activity we measured their dose-dependent effects on *Staphylococcus aureus*.

2. Results and discussion

In our previous synthesis of **1e** we prepared the *N*-methylated and *O*-TIPS protected benzyl threoninate **6**, required for the cyclisation with ylide Ph_3PCCO **2** to give tetramic acid **8**, from L-threonine in seven steps, which included N-nosylation to enable *N*-mono-methylation.[4] Although we still deem this a viable approach,

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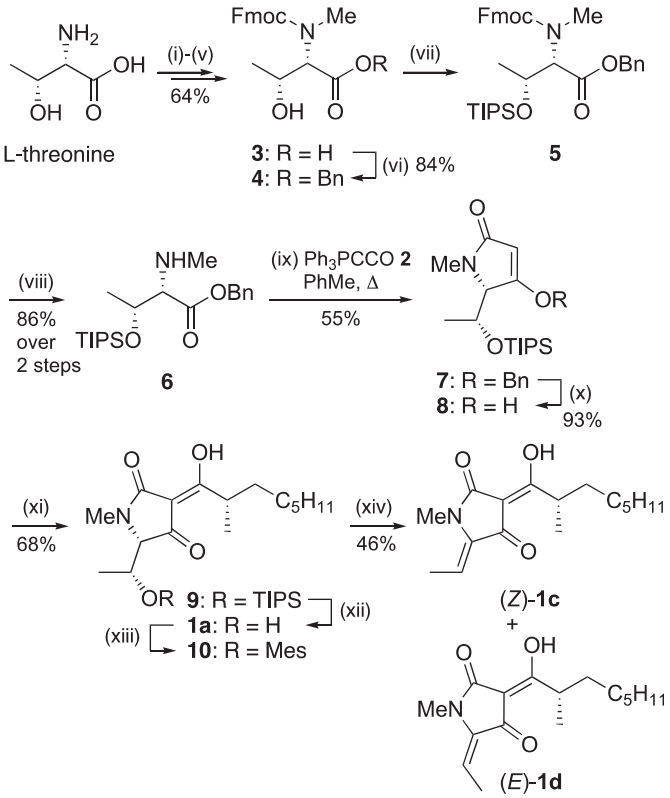
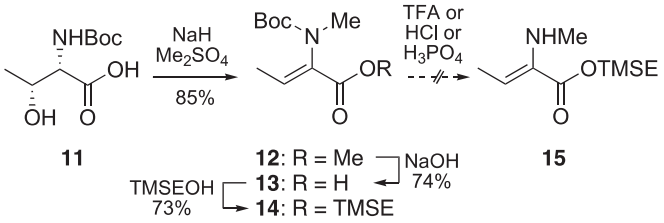
we now explored a new strategy for the synthesis of ester **6** that achieves *N*-mono-methylation without preceding nosylation. The yield of this route to **6** is higher (46%) than that of the previous one (34%), it is upscalable since its intermediates are easier to purify without columning, and it is more atom-economical. We assumed that the unknown configuration at C-9 of **1c** and **1d** would be *S* as in the case of penicillenols A and C.

According to literature[5] L-threonine was converted in five steps and 64% yield to its *N*-methyl-*N*-Fmoc derivative **3** (Scheme 1). This sequence was comprised of O-acetylation and *N*-Fmoc protection of threonine, a subsequent oxazolidinone formation with paraformaldehyde, and the reduction of the latter to give the *N*-methyl derivative. Hydrolysis left acid **3**, which was esterified with benzyl bromide and Cs_2CO_3 yielding the hitherto unknown ester **4** as colourless crystals in over 80%. *O*-TIPS protection afforded **5**, the Fmoc group of which was removed with piperidine to leave the amino ester **6** in 86% over the last two steps, which can be carried out in one pot. Refluxing amino ester **6** with ylide **2** in toluene gave benzyl tetramate **7** in 55% yield via a domino sequence comprised of amine addition to the C=C bond of **2** followed by an intramolecular Wittig olefination of the so-formed amide ylide. Hydrogenolysis of **7** led to tetramic acid **8**, which was 3-acylated according to the Yoda protocol[3] with *S*-2-methyloctanoic acid[6] prepared by α-methylation of octanoic acid using an Evans auxiliary. Pure tetramic acid **9** was obtained in 68% yield upon chromatography of the crude on a reversed stationary phase (Merck LiChrorep RP-18). Its desilylation with 70% HF/pyridine and subsequent purification by another RP column chromatography furnished penicillenol A_1 **1a** in 88% yield. Mesylation of the 6-OH of **1a** gave **10**, which was treated overnight with 0.1 N aqueous NaOH/THF to give a 3:1 mixture of both penicillenols B. It was base-line separated on a prep HPLC column (Kinetex 5 μm, C18, 250×21.2 mm) to afford the pure isomers **1c** (35%) and **1d** (11%), each as a 5:6 mixture of two *exo*-enol tautomers.



Scheme 1. Reagents and conditions: (i) 28 equiv AcCl/AcOH, 6 N HCl 1:1, 0 °C, 45 min; (ii) FmocONSu, NEt_3, dioxane/THF 1:1, 12 h, rt; (iii) $HO(CH_2O)_nH$, *p*TsOH, tol., 1 h, refl.; (iv) $AlCl_3$, Et_3SiH, CH_2Cl_2, 4 h, rt; (v) dioxane/2 M aq HCl 1:1, 60 °C, 12 h; (vi) Cs_2CO_3, BnBr, rt; (vii) TIPSOTf, NEt_3, 12 h, 0 °C; (viii) 20% piperidine, CH_2Cl_2; (ix) 1.2 equiv **2**, tol., refl., 16 h; (x) 1 bar H_2, Pd/C, MeOH; (xi) *S*-2-methyloctanoic acid, 1.2 equiv EDC, 0.2 equiv DMAP, CH_2Cl_2, 12 h, rt, then 1.5 equiv $CaCl_2$, 1.5 equiv NEt_3, rt, 12 h, then 3.5 h refl.; (xii) 8 equiv HF/pyridine (88%), THF; (xiii) 2 equiv MesCl, 4 equiv NEt_3, 0.4 equiv DMAP; (xiv) THF, 0.1 N NaOH 2:1, rt.

Originally, we intended to introduce the $C^5{=}C^6$ bond early in the synthesis of penicillenols B to avoid the necessity of protecting the secondary alcohol of threonine. *N*-Boc-L-threonine **11** gave the *N*-methylated aminobutenoate **12** in one step and as a 3:1-mixture of *Z*- and *E*-isomers when treated with NaH and Me_2SO_4 (Scheme 2). However, all attempts at cleaving the Boc group to liberate unsaturated amino esters such as **15**, required for the cyclisation with ylide **2**, failed for unknown reasons. So this approach had to be abandoned.



Scheme 2. TMSE=$Me_3SiCH_2CH_2$.

The configurations of the $C^5{=}C^6$ bond of our synthetic penicillenols B were assigned by NOESY experiments (cf. Supplementary data). For the assignment of the configuration at C-9 of the natural penicillenols B we compared the known chemical shifts in their ^{13}C NMR spectra[1] with those of our synthetic (9*S*)-configured *Z*- and *E*-diastereomers. Table 1 shows a perfect match of indicative carbon signals. The specific rotations of our samples of penicillenols B were $[\alpha]_D^{24}$ −15 (*c* 0.23, MeOH) for **1c** and $[\alpha]_D^{24}$ −6 (*c* 0.13, MeOH) for **1d**, which is the other way round as had been reported in literature[1] (**1c**: $[\alpha]_D^{24}$ −7.8; **1d**: $[\alpha]_D^{24}$ −15.9). Given the matching NMR spectra, the congruence of the HPLC retention times (*Z*-products eluted first in our hands and in literature), and the fact that **1a** did not racemise under the basic conditions of the final elimination step, we assume a mixing up of samples or data by the isolating group.

This group also reported the cytotoxicities of penicillenols A–C in MTT assays[7] against four cancer cell lines of which only leukaemia HL-60 responded to penicillenols B. Since these activities lay within the range of clinical applicability we tested **1c** and **1d** for growth inhibition of cells of highly invasive 518A2 melanoma and cisplatin-resistant HT-29 colon carcinoma and of the hybrid endothelial cell line Ea.hy926. Both compounds are equally active and even exceed the effect of cisplatin against HT-29. However, they are still far from clinical usability (Table 2).

The penicillenols B also showed dose-dependent antimicrobial activity against Gram-positive bacteria such as *S. aureus* (Fig. 2) and Gram-negative bacteria such as the export-limited *Escherichia coli* tolC mutant (not shown). Interestingly, penicillenol B_1 was distinctly more active than penicillenol B_2.

3. Conclusion

We reported a new synthetic route to penicillenol A_1 and, by mesylation–elimination of this, also the first access to both penicillenols B_1 and B_2. We assigned the stereochemistry of C-9 in the 3-acyl sidechain as *S*. The penicillenols B were efficacious against two cancer cell lines and they also showed antimicrobial activity against Gram-positive and -negative bacteria. It is worthy of note that penicillenol B_1 was conspicuously more active than its B_2

Table 1
Chemical shifts δ[a] [ppm] of indicative hydrogen and carbon atoms of natural and synthetic penicillenols B_1 and B_2

Position	Nat **1c**[1] *exo*^{A}/*exo*^{B}	Synt **1c** *exo*^{A}/*exo*^{B}	Nat **1d**[1] *exo*^{A}/*exo*^{B}	Synt **1d** *exo*^{A}/*exo*^{B}
6 (^{1}H)	5.89, q, *J* 7.7	5.87, q, *J* 8.0	5.38, q, *J* 7.8	5.38, q, *J* 7.7
7 (^{1}H)	2.03, d, *J* 8.2	2.01, d, *J* 8.0	2.23, d, *J* 7.8	2.23, d, *J* 7.7
17 (^{1}H)	3.39, s	3.37, s	2.99, s	3.04, s
2 (^{13}C)	172.5/165.7	172.5/165.6	170.8/164.0	170.9/164.1
3 (^{13}C)	99.6/101.8	99.5/101.8	101.3/103.7	101.3/103.7
4 (^{13}C)	191.2/197.4	191.1/197.4	190.8/196.6	190.8/196.6
5 (^{13}C)	136.7	136.7	134.2	134.3
6 (^{13}C)	107.5	107.3	111.8	111.8
8 (^{13}C)	181.0/185.0	180.9/184.8	182.8/187.3	183.1/187.3
17 (^{13}C)	27.9	27.8	24.7	24.7/24.9

[a] ^{13}C NMR spectra were recorded in $CDCl_3$ at 150 MHz (natural **1c**,**d**)[1] and 75 MHz (synthetic **1c**,**d**). ^{1}H NMR spectra were recorded in $CDCl_3$ at 600 MHz (natural **1c**,**d**)[1] and 300 MHz (synthetic **1c**,**d**).

Table 2
Inhibitory concentrations IC_{50} (72 h)[a] in μM of penicillenols B_1 **1c** and B_2 **1d** when applied to 518A2 melanoma, HT-29 colon carcinoma, and Ea.hy926 hybrid endothelial cells

Cell line/compound	518A2	HT-29	Ea.hy926
1c	24.7±3.1	28.0±1.1	32.7±2.2
1d	21.1±1.3	26.0±1.8	32.6±4.0
Cisplatin	9.0±2.0	>100	—

[a] Values are derived from concentration–response curves obtained by measuring the percentage absorbance of viable cells relative to untreated controls (100%) after 72 h exposure in MTT assays. Values represent means of four independent runs±SD.

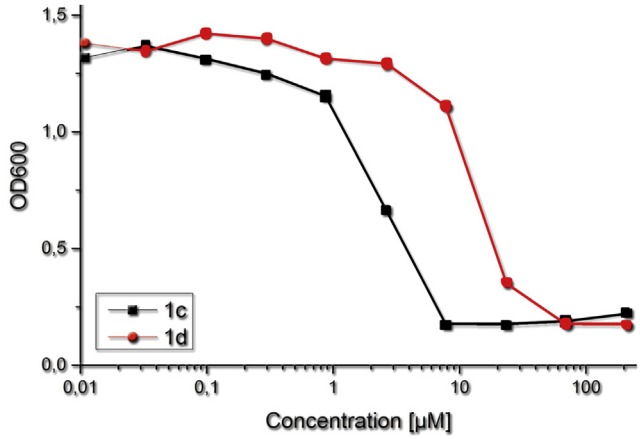


Fig. 2. Dose-dependent antimicrobial effects of penicillenols B_1 **1c** and B_2 **1d** on *Staphylococcus aureus*. Bacterial growth was measured as 'turbidity' (OD at 600 nm). Minimal inhibitory concentrations (MIC) were ca. 8 μM for **1c** and 70 μM for **1d**.

congener against the bacteria selected by us. A more comprehensive screening for antimicrobial activity is currently underway.

4. Experimental

4.1. General

Melting points (uncorrected): Büchi melting point apparatus M-565. IR: Perkin–Elmer Spectrum 100 FT-IR spectro-photometer with ATR sampling unit. NMR: if not indicated otherwise, measured in $CDCl_3$ at 300 MHz (^{1}H) or 75.5 MHz (^{13}C) on a Bruker Avance 300 spectrometer. Chemical shifts are given in parts per million (δ) downfield from Me_4Si as internal standard for ^{1}H and ^{13}C. Mass spectra: Finnigan MAT 8500 (EI, 70 eV). HRMS: UPLC/Orbitrap MS system in ESI mode. Optical rotations: Perkin–Elmer Polarimeter 343 (λ=589 nm). For column chromatography Merck silica gel 60 (230–400 mesh) or Merck LiChrorep RP-18 (40–63 μm) was used. HPLC columns: Phenomenex Kinetex 5 μ C18 250×4.6 mm, flow rate 0.7 mL/min (analyt.) and 250×21.2, 14.9 mL/min (prep.). Solvents were dried and distilled (diethyl ether, toluene and THF over Na, CH_2Cl_2 over CaH_2) and stored under an atmosphere of dry argon. Starting compounds were bought from the usual sources and used without further purification. For the synthesis of compounds **12**–**14** see Supplementary data.

4.2. Syntheses and characterisation

*4.2.1. Benzyl (2S,3R)-N-[(9H-fluoren-9-yl)methoxycarbonyl]-N-methyl-threoninate (**4**).* Analogously to a general protocol[8] a solution of *N*-Fmoc-*N*-methyl-L-threonine (**3**)[5] (6.90 g, 19.5 mmol) in methanol (100 mL) was treated with Cs_2CO_3 (3.25 g, 10.0 mmol) and stirred at room temperature until it had completely dissolved. The solvent was evaporated and the remainder was suspended twice in $CHCl_3$ and evaporated each time under reduced pressure to remove residual water. The resulting solid was treated with DMF (300 mL) and benzylbromide (2.6 mL, 22.0 mmol) and the mixture thus obtained was vigorously stirred for 12 h and then evaporated under reduced pressure. The residue was partitioned between diethyl ether and water, the aqueous phase was extracted twice with diethyl ether, and the combined organic layers were finally washed with brine, dried over Na_2SO_4, and evaporated in vacuum. The crude product was purified by column chromatography (silica gel; hexane/ethyl acetate, 10:1 to 3:1) to afford ester **4** (7.25 g, 16.3 mmol, 84%) as white needles of mp 91 °C; R_f 0.20 (hexane/ethyl acetate, 3:1); $[\alpha]_D^{20}$ −2.0 (*c* 1.00, CH_2Cl_2); ν_{max} 3481, 3035, 2961, 1742, 1699, 1479, 1451, 1401, 1373, 1308, 1236, 1195, 1146, 1104, 1035, 988, 879, 757, 738, 697 cm^{-1}; δ_H ($CDCl_3$) 1.22 (3H, d, J_{HH} 6.5 Hz, CCH_3), 3.02 (3H, s, NCH_3), 4.27 (1H, t, J_{HH} 7.0 Hz, FmocCH), 4.45 (2H, d, J_{HH} 7.0 Hz, $FmocCH_2$), 4.50 (1H, dq, J_{HH} 4.9, 6.5 Hz, 3-H), 4.66 (1H, d, J_{HH} 4.9 Hz, 2-H), 5.19 (1H, d, J_{HH} 12.4 Hz, CH*H*^{a}Ph), 5.26 (1H, d, J_{HH} 12.4 Hz, CH*H*^{b}Ph), 7.29–7.37 (7H, m, ArH), 7.41 (2H, t, J_{HH} 7.4 Hz, FmocArH), 7.60 (2H, d, J_{HH} 7.4 Hz, FmocArH), 7.78 (2H, d, J_{HH} 7.7 Hz, FmocArH); δ_C ($CDCl_3$) 19.5 (C-4), 33.9 (NMe), 47.1 (Fmoc*C*H), 64.7 (C-2), 67.0 (CH_2Ph), 67.1 (C-3), 67.9 (FmocCH_2), 119.8, 124.9, 126.9, 127.6, 128.1, 128.3, 128.5, 135.2, 141.2, 143.7, 157.5, 169.8. HRMS: *m*/*z* calcd for [M+H, $C_{27}H_{28}NO_5^+$]: 446.19620; found: 446.19553.

*4.2.2. Benzyl (2S,3R)-N-methylthreoninate (**6**).* A solution of benzyl *N*-Fmoc-*N*-methyl-threoninate **4** (2.98 g, 6.7 mmol) in 60 CH_2Cl_2 (60 mL) was kept under argon atmosphere at 0 °C and treated with NEt_3 (1.1 mL, 810 mg, 8.0 mmol) and triisopropylsilyltriflate (1.98 mL, 2.25 g, 7.4 mmol). The mixture was allowed to warm to room temperature and stirred until the reaction was complete by TLC (hexane/ethyl acetate, 3:1). The solution was cooled to 0 °C, treated with piperidine (18 mL), stirred for 10 min at 0 °C, and

eventually concentrated in vacuo at 25 °C. The remaining piperidine solution was immediately loaded onto a column and separated (silica gel; hexane/ethyl acetate, 20:0 to 20:1) to afford ester **6** (2.16 g, 5.7 mmol, 86% after two steps) as a colourless oil. R_f=0.63 (cyclohexane/EtOAc, 2:1, +1% NEt_3). $[\alpha]_D^{20}$ 2.2 (*c* 1.00, CH_2Cl_2); ν_{max} 2942, 2895, 2866, 1742, 1732, 1463, 1456, 1373, 1212, 1152, 1124, 1099, 1060, 1014, 998, 967, 949, 918, 882, 746, 733, 696, 676 cm^{-1}; δ_H ($CDCl_3$) 0.95–1.07 (21H, m, TIPS), 1.26 (3H, d, J_{HH} 6.3 Hz, CCH_3), 1.71 (1H, br s, NH), 2.40 (3H, s, NCH_3), 3.14 (1H, J_{HH} 3.6 Hz, CHN), 4.32 (1H, qd, J_{HH} 6.3, 3.6 Hz, C*H*CH_3), 5.10 (1H, d, J_{HH} 12.2 Hz, CH_2^aPh), 5.22 (1H, d, J_{HH} 12.2 Hz, CH^b_2Ph), 7.27–7.40 (5H, m, ArH); δ_C ($CDCl_3$) 12.6 (SiCH), 18.0 ($SiCCH^a_3$), 18.1 ($SiCCH^b_3$), 20.5 ($CHCH_3$), 35.3 (NMe), 66.4 (CH_2), 69.4 ($CHCH_3$), 69.9 (*C*HN), 128.2 (ArH), 128.4 (ArH), 128.5 (ArH), 135.8 (ArC^q), 173.3 (CO).

*4.2.3. 3-[1′-Hydroxy-(2′S)-methyl-oct-1′-ylidene]-(5S)-[(1′R)-triisopropylsilyloxy-ethyl]-1-methylpyrrolidine-2,4-dione (**9**).* To a solution of (*S*)-2-methyloctanoic acid[6] (0.33 g, 2.1 mmol) in CH_2Cl_2 (20 mL) at room temperature were added EDC·HCl (0.48 g, 2.5 mmol), tetramic acid **8**[4] (0.55 g, 1.75 mmol), and DMAP (0.05 g, 0.4 mmol), and the resulting mixture was stirred for 12 h. NEt_3 (0.62 mL, 4.5 mmol) and dry $CaCl_2$ (0.29 g, 2.6 mmol) were added and stirring was continued for 24 h. After dilution with 20 mL of CH_2Cl_2 the organic phase was washed with 1 M $NaHSO_4$ and 0.05 M Na_2-EDTA solutions. The aqueous phases were re-extracted with CH_2Cl_2 and the combined organic layers were dried and concentrated in vacuum. The crude product was purified by a reversed phase silica gel column (22×1.2 cm, rinsing with 85% MeOH, then elution with 100% MeOH) to afford 540 mg (1.19 mmol, 68%) of **9** as a red oil (analytic HPLC: 85% MeOH, after 15 min in 10 min to 100% with 0.1% HCOOH in the water fraction; t_R of product (3-acyl tetramic acid): 31.2 min, λ_{max}=285 nm; t_R of 4-*O*-acyl tetramate: 29.5 min, λ_{max}=226 nm); $[\alpha]_D^{20}$ −39.1 (*c* 1.00, MeOH); ν_{max} 2930, 2867, 1710, 1652, 1615, 1463, 1376, 1328, 1262, 1214, 1139, 1096, 1069, 999, 971, 922, 882, 797, 755, 703, 677 cm^{-1}; δ_H ($CDCl_3$) 0.84 (3H, t, J_{HH} 6.6 Hz, CH_2CH_3), 0.96–1.01 (21H, m, TIPS), 1.11 (3H, d, J_{HH} 6.7 Hz, C-16), 1.19–1.31 (8H, m), 1.40–1.50 (1H, m, 10-H^a), 1.43 (3H, d, J_{HH} 6.9 Hz, C-7), 1.59–1.70 (1H, m, 10-H^b), 3.10 (3H, s, NMe), 3.47 (1H, d, J_{HH} 1.6 Hz, 5-H), 3.57 (1H, ddqui, J_{HH} 6.3, 1.9, 6.7 Hz, 9-H), 4.56 (IH, dq, J_{HH} 1.6, 6.9 Hz, 6-H); δ_C ($CDCl_3$) 12.6 (SiCH), 14.0 (C-15), 16.7 (C-16), 17.9 ($SiCCH_3$), 22.5 (CH_2), 22.9 ($CHCH_3$), 27.1 (CH_2), 29.0 (NMe), 29.2 and 31.6 (CH_2), 34.0 (C-10), 35.8 (C-9), 68.0 (*C*HCH_3), 72.4 (C-5), 101.1 (C-3), 174.4 (CON), 190.6, 193.2. MS (EI), *m*/*z* (%): 410 (100) $C_{22}H_{40}NO_4Si^+$, 366 (27), 253 (29) $C_{15}H_{22}NO_3^+$, 201 (28) $C_{11}H_{25}OSi^+$, 157 (24). HRMS: *m*/*z* calcd for [M+H, $C_{25}H_{48}NO_4Si^+$]: 453.3347, found: 453.3341.

*4.2.4. Penicillenol B_1, (5Z,2′S)-5-ethylidene-3-(1-hydroxy-2-methyloct-1-ylidene)-1-methylpyrrolidine-2,4-dione (**1c**) and penicillenol B_2, (5E,2′S)-5-ethylidene-3-(1-hydroxy-2-methyloct-1-ylidene)-1-methylpyrrolidine-2,4-dione (**1d**).* Penicillenol A_1 (310 mg, 1.04 mmol; for its synthesis, data, and spectra cf. Supplementary data) was dissolved in THF, treated with NEt_3 (580 μL, 4.2 mmol, 4 equiv), methanesulfonylchloride (170 μL, 4.2 mmol, 4 equiv), and DMAP (51 mg, 0.4 mmol, 0.4 equiv) and stirred for 12 h. The mixture was poured into water, acidified with $NaHSO_4$ and extracted three times with ethyl acetate. The combined organic layers were washed with brine, dried over Na_2SO_4 and concentrated in vacuum to leave crude product **10**. It was dissolved in THF (60 mL), 0.1 N aqueous NaOH (30 mL) was added, and the resulting solution was stirred at room temperature for 3 d. $NaHSO_4$ was added, the resulting mixture was extracted three times with ethyl acetate, and the organic layers were washed with brine, dried over Na_2SO_4 and evaporated in vacuum. The remainder was purified by HPLC (70% MeOH/0.1% HCOOH) to afford 100 mg (0.35 mmol, 35% over two steps) of penicillenol B_1 as a red oil in the first fraction and 29 mg (0.17 mmol, 11% over two steps) of penicillenol B_2 in the second fraction. Analytic HPLC: elution with 85% MeOH/0.1% HCOOH for 10 min, then gradient rise to 95% MeOH within 3 min; t_R of **1c**: 16.3 min, λ_{max}=270 nm, t_R of **1d**: 17.8 min, λ_{max}=270 nm.

*4.2.4.1. Penicillenol B_1 (**1c**).* $[\alpha]_D^{24}$ −15 (*c* 0.23, MeOH); ν_{max} 2927, 2857, 1705, 1676, 1610, 1450, 1407, 1370, 1341, 1322, 1239, 1217, 1170, 1111, 1093, 1031, 997, 903, 826, 789, 726, 618, 584, 574, 562 cm^{-1}; δ_H ($CDCl_3$, *6:5 mixture of tautomer*[a] *and tautomer*[b]) 0.79–0.89 (3H, m, 15-H), 1.15 (3H, d, J_{HH} 6.9 Hz, 16-H), 1.19–1.33 (8H, m, CH_2^{a+b}), 1.38–1.52 (1H, m, 10-H), 1.60–1.75 (1H, m, 10-H), 2.01 (3H, d, J_{HH} 8.0 Hz, 7-H^a), 2.07 (3H, d, J_{HH} 8.0 Hz, 7-H^b), 3.34 (3H, s, NMe^b), 3.37 (3H, s, NMe^a), 3.62–3.75 (1H, m, 9-H), 5.85 (1H, q, J_{HH} 8.0 Hz, 6-H^a), 5.87 (1H, q, J_{HH} 8.0 Hz, 6-H^b); δ_C ($CDCl_3$) 11.4 (C-7^a), 11.8 (C-7^b), 14.0 (C-$15^{a/b}$), 17.0 (C-16^b), 17.2 (C-16^a), 22.5 (C-$13^{a/b}$), 27.1 (C-11), 27.8 (C-17), 29.2 (C-12^a), 29.25 (C-12^b), 31.6 (C-14), 33.3 (C-10^b), 33.7 (C-10^a), 35.8 (C-9^a), 37.4 (C-9^b), 99.5 (C-3^a), 101.8 (C-3^b), 107.3 (C-6^a), 108.1 (C-6^b), 134.4 (C-5^b), 136.7 (C-5^a), 165.6 (C-2^b), 172.5 (C-2^a), 180.9 (C-8^a), 184.8 (C-8^b), 191.1 (C-4^a), 197.4 (C-4^b); MS (EI), *m*/*z* (%) 279 (95, M^+), 251 (7), 232 (8), 222 (12), 208 (76), 195 (99), 177 (16), 166 (100), 139 (42), 127 (4), 113 (6), 86 (15), 70 (30), 57 (25), 44 (21); HRMS: *m*/*z* calcd for [M+H, $C_{16}H_{26}NO_3^+$]: 280.19072, found: 280.19022.

*4.2.4.2. Penicillenol B_2 (**1d**).* $[\alpha]_D^{24}$ −6 (*c* 0.13, MeOH); ν_{max} 2928, 2858, 2043, 2003, 1703, 1671, 1615, 1445, 1398, 1374, 1341, 1315, 1278, 1206, 1164, 1091, 1050, 883, 826, 802, 726, 681, 606, 584, 573 cm^{-1}; δ_H ($CDCl_3$, *6:5 mixture of tautomer*[a] *and tautomer*[b]) 0.81–0.90 (3H, m, 15-H), 1.19 (3H, d, J_{HH} 7.0 Hz, 16-H), 1.22–1.29 (8H, m, $CH_2^{a/b}$), 1.42–1.51 (1H, m, 10-H), 1.65–1.75 (1H, m, 10-H), 2.23 (3H, d, J_{HH} 7.7 Hz, 7-H^{a+b}), 3.04 (3H, s, NMe^b), 3.05 (3H, s, NMe^a), 3.70 (1H, *pseudo*-sxt, J_{HH} 7.0 Hz, 9-H), 5.38 (1H, q, J_{HH} 7.7 Hz, 6-H^a), 5.46 (1H, q, J_{HH} 7.7 Hz, 6-H^b); δ_C ($CDCl_3$) 11.8 (C-7^a), 12.4 (C-7^b), 14.0 (C-$15^{a/b}$), 17.0 (C-16^b), 17.2 (C-16^a), 22.6 (C-$13^{a/b}$), 24.7 (C-17^a), 24.9 (C-17^b), 27.2 (C-$11^{a/b}$), 29.3 (C-$12^{a/b}$), 31.6 (C-$14^{a/b}$), 33.6 (C-$10^{a/b}$), 35.7 (C-9^a), 37.1 (C-9^b), 101.3 (C-3^a), 103.7 (C-3^b), 111.8 (C-6^a), 112.6 (C-6^b), 134.3 (C-5^a), 136.0 (C-5^b), 164.1 (C-2^b), 170.9 (C-2^a), 183.1 (C-8^a), 187.3 (C-8^b), 190.8 (C-4^a), 196.6 (C-4^b); *m*/*z* (%) 279 (99, M^+), 251 (7), 232 (8), 222 (12), 208 (66), 195 (99), 177 (15), 166 (100), 139 (35), 113 (4), 85 (7), 70 (24), 57 (14), 44 (13); HRMS: *m*/*z* calcd for [M+H, $C_{16}H_{26}NO_3^+$]: 280.19072, found: 280.19040.

4.3. Cell cultures and growth inhibition (MTT) assay

4.3.1. Cell cultures. The human 518A2 melanoma cells (Department of Radiotherapy and Radiobiology, University Hospital Vienna)[9] and the human colon adenocarcinoma cell line HT-29 (DSMZ no.: ACC 299) were grown in Dulbecco's Modified Eagle's Medium (DMEM) or Roswell Park Memorial Institute (RPMI) Medium (HT-29), supplemented with 10% foetal bovine serum (FBS), 1% antibiotic-antimycotic solution (all from Gibco) and 250 μg/mL gentamycin (SERVA). The endothelial hybrid cell line Ea.hy926 (ATCC no.: CRL-2922)[10] was grown in conditioned DMEM with an endothelial medium supplement (PAA/GE Healthcare) added. The cells were maintained in a moisture-saturated atmosphere (5% CO_2, 95% humidity) at 37 °C.

4.3.2. MTT assay.[7] MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium hydrobromide; ABCR] was used to identify the metabolic activity of vital cells, which are capable of reducing it to a violet formazan. 518A2, HT-29, and Ea.hy926 cells (5×10^4/mL) were seeded and cultured for 24 h in 96-well microplates. Incubation of cells following treatment with the test

compounds was continued for 72 h. Blank and solvent controls were treated identically. Then, MTT in phosphate-buffered saline was added to a final concentration of 0.05% (518A2, Ea.hy926) or 0.1% (HT-29). After 2 h the precipitate of formazan was dissolved in DMSO containing 10% sodium dodecylsulfate. The microplates were swiftly turned to discard the medium before adding the solvent mixture. The microplates were gently shaken in the dark for 30 min and absorbances at 570 nm and 630 nm (background) were measured with a Tecan F-200 plate reader. All experiments were carried out in quadruplicate, and the percentage of the viable cells was calculated with Origin 8.1G as the mean±standard deviation relative to the control (100%).

4.4. Antibacterial activity test

S. aureus (NCTC 8325) was cultivated in T-medium, which was composed of tryptone (LP 0042, 17 g/L), neutralised soya peptone (LP 0044, 3 g/L), NaCl (0.1 M), KCl (2 mM), MOPS (50 mM), $CaCl_2 \cdot 2H_2O$ (2 mM) and glucose (1%). Medium (200 mL) was inoculated with an *S. aureus* colony and incubated at 37 °C with shaking overnight. An aliquot of the overnight culture was diluted with fresh T-medium to obtain 50 mL with a start OD_{600} of 0.01. This culture was allowed to grow for another 3 h at 37 °C. The resulting culture was added to the wells of a transparent 96-well microtiter plate containing diluted DMSO solutions of the test compounds. The total volume in each plate was 180 μL. The bacteria were incubated with the compounds at 37 °C for 21 h. Bacterial growth was followed via determination of the optical density (OD) at 600 nm (turbidity). Blank and solvent controls were included on the microtiter plate.

Supplementary data

Syntheses and data of compounds **1a**, **12**, and **14**; NMR spectra of **1a**, **1c**, **1d**, **4**, **6**, **9**, **12**, and **14**. Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.tet.2015.05.116.

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